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Adenylate Cyclase and Cyclic AMP Phosphodiesterase in *Bradyrhizobium japonicum* Bacteroids†

CAROL A. CATANESE,‡ DAVID W. EMERICH,* AND WARREN L. ZAHLER

Department of Biochemistry, University of Missouri-Columbia, Columbia, Missouri 65211

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Adenylate cyclase and cyclic AMP (cAMP) phosphodiesterase have been identified and partially characterized in bacteroids of *Bradyrhizobium japonicum* 3I1b-143. Adenylate cyclase activity was found in the bacteroid membrane fraction, whereas cAMP phosphodiesterase activity was located in both the membrane and the cytosol. In contrast to other microorganisms, *B. japonicum* adenylate cyclase remained firmly bound to the membrane during treatment with detergents. Adenylate cyclase was activated four- to fivefold by 0.01% sodium dodecyl sulfate (SDS), whereas other detergents gave only slight activation. SDS had no effect on the membrane-bound cAMP phosphodiesterase but strongly inhibited the soluble enzyme, indicating that the two enzymes are different. All three enzymes were characterized by their kinetic constants, pH optima, and divalent metal ion requirements. With increasing nodule age, adenylate cyclase activity increased, the membrane-bound cAMP phosphodiesterase decreased, and the soluble cAMP phosphodiesterase remained largely unchanged. These results suggest that cAMP plays a role in symbiosis.

Cyclic AMP (cAMP; adenosine 3′,5′-monophosphate) and the enzymes involved in its metabolism, adenylate cyclase and cAMP phosphodiesterase, have been identified in a large number of organisms, where they participate in the regulation of a variety of processes. In higher organisms, cAMP is a second messenger used by many hormones in regulating cellular metabolism. As key enzymes in this system, the regulatory properties of adenylate cyclase, which catalyzes the conversion of ATP to cAMP, and cAMP phosphodiesterase, which hydrolyzes cAMP to AMP, have been studied extensively. In contrast, adenylate cyclase and cAMP phosphodiesterase in bacteria have been less well characterized, and the role most commonly associated with cAMP in procaryotes is the regulation of gene expression via the process of catabolite repression (19, 20, 30). cAMP regulates gene expression by binding to a receptor protein, CAP, and the cAMP-CAP complex in turn then binds to specific regions of the DNA and promotes transcription of those operons for catabolic enzymes.

There are several features of *Bradyrhizobium* and *Rhizobium* spp. which may show involvement of cAMP. These are its catabolite repression behavior, regulation during symbiosis of ammonium assimilation, and morphological differentiation. *Bradyrhizobium japonicum* is a dimorphic organism which in the free-living form can grow heterotrophically on a number of carbon sources (28) or chemoautotrophically on H_2 and CO_2 (9) and in the symbiotic form, in association with soybean, is capable of biological nitrogen fixation (28). A number of investigators have reported catabolite repression in free-living *Rhizobium* spp. (7, 13, 16, 17, 21, 25). However, only McGetrick et al. (17) and Lim and Shanmugam (13) have studied the effect of exogenous cAMP on catabolite repression. In *Rhizobium meliloti*, McGetrick et al. (17) found that neither exogenous cAMP nor additional copies of the adenylate cyclase gene from *R. meliloti*, carried on plasmid pRK290, could alleviate repression of formate-dependent CO_2 fixation. Lim and Shanmugam (13) reported that exogenous cAMP alleviated the malate-mediated repression of hydrogen uptake in *B. japonicum* and concluded that cAMP plays a major role in the regulation of hydrogen metabolism. McGetrick et al. (17) showed that the malate-mediated repression of hydrogen uptake in *B. japonicum* could be significantly decreased in cells carrying the *R. meliloti* adenylate cyclase gene on plasmid pRK290. Symbiotic nitrogen fixation is an energy-demanding process and relies entirely upon the host plant to provide the carbon compounds necessary to meet its energy needs. Thus, Guerinot and Chelm (8) reasoned that cAMP may regulate nitrogen fixation in response to the availability of carbon compounds.

Upchurch and Elkan (26) reported that cAMP represses the formation of enzymes involved in ammonium assimilation in cultures of *B. japonicum*. During nodule formation, the symbiotic forms of rhizobium, referred to as bacteroids, derepress nitrogenase, the enzyme that reduces atmospheric dinitrogen to ammonium, and represses ammonium assimilatory enzymes. These results imply that cAMP may regulate nitrogen fixation via repression of the ammonium-assimilatory enzymes.

cAMP has been shown to be involved in morphological variations in some procaryotes (2). During symbiotic development, the rhizobium undergoes numerous morphological and biochemical changes to the nitrogen-fixing bacteroid form. cAMP may perform a role in symbiotic differentiation.

No measurements of cAMP have been reported for *Rhizobium* bacteroids, nor has adenylate cyclase or cAMP phosphodiesterase been measured in either the bacteroid or the free-living form. Here, we report the initial characterization of adenylate cyclase and two forms of cAMP phosphodiesterases in *B. japonicum* bacteroids.

MATERIALS AND METHODS

Preparation of bacteroid samples. Soybean plants (*Glycine max* L. cv. Williams 82) were inoculated with *B. japonicum* (strain 3I1B-143) and grown in a greenhouse with a nitrogen-free nutrient solution (11). Plants were harvested between 4

* Corresponding author.

† Contribution no. 10,830 of the Agricultural Experiment Station of the University of Missouri.

‡ Present address: Department of Pharmacology, ICI Pharmaceuticals Group, Wilmington, DE 19897.

and 5 weeks after inoculation, and the root nodules were removed for bacteroid isolation.

The isolation procedure was basically as described by Karr et al. (11), with the following isolation buffer: 50 mM potassium phosphate, pH 7.0, 5 mM $MgCl_2$, and 1 mM EDTA. All isolation steps were performed at 0 to 4°C. Nodules were homogenized in a Waring blender; for each gram of nodules, 10 ml of isolation buffer containing 17% (wt/vol) sucrose and 0.33 g of polyvinylpyrrolidone (PVP) was used. After being filtered through cheesecloth, the homogenate was centrifuged at 400 × *g* for 10 min, and the pellet was discarded. The supernatant was then centrifuged at 8,000 × *g* for 20 min, and the resulting pellet was suspended in 2 ml of isolation buffer containing 17% (wt/vol) sucrose per g of nodules.

The crude bacteroid suspension was layered on a discontinuous sucrose gradient, consisting of 6 ml of 57% (wt/wt) sucrose, 5 ml of 40% (wt/wt) sucrose, and 10 ml of 30% (wt/wt) sucrose, and centrifuged at 72,000 × *g* for 35 min in a Beckman SW28 rotor. The bacteroid layer was collected from the 40 to 57% sucrose interface and diluted with 2 volumes of isolation buffer. This suspension was then layered on a gradient of 10 ml of 40% and 10 ml of 30% sucrose and centrifuged at 72,000 × *g* for 35 min. The pellet, containing the bacteroids, was then washed by suspension in distilled water and centrifuged at 8,000 × *g* for 20 min.

To disrupt the bacteroids, the washed pellet was suspended in 0.5 ml of isolation buffer per g of nodules containing 1 M glycerol and 1 mM dithiothreitol and then ruptured by passage through a French pressure cell at 16,000 lb/in^2. Preparations of disrupted bacteroids were stored frozen at −70°C in portions of 1 to 2 ml prior to assay for enzymatic activity.

Adenylate cyclase assay. Adenylate cyclase activity was measured in a reaction mixture containing, in a final volume of 0.1 ml, 10 mM $MgCl_2$, 1 mM ATP (containing 0.5×10^6 to 1.0×10^6 cpm of [α-^{32}P]ATP), 4 mM creatine phosphate, 2.5 U of creatine phosphokinase, 1 mM cAMP (containing approximately 10,000 cpm of [^{3}H]cAMP), and 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.5). Unless otherwise indicated, the reaction was initiated by addition of 25 μl of enzyme and incubated at 30°C for 10 min. The reaction was stopped by the addition of 1 ml of 0.17 M $HClO_4$. Product cAMP was separated by sequential chromatography on Dowex and alumina columns as described by Cooper and Londos (5). The enzymatic reaction was linear with time for at least 20 min, and recovery of [^{3}H]cAMP ranged from 65 to 75%. Each data point represents the average of triplicate determinations.

Phosphodiesterase assay. The hydrolysis of cAMP was measured with an assay mixture which contained 6 mM $MgCl_2$ and 1 mM cAMP (containing about 50,000 cpm of [^{3}H]cAMP). For most experiments, the reaction was buffered at the pH indicated with a mixture of 50 mM each MOPS (morpholinepropanesulfonic acid), MES (morpholinoethanesulfonic acid), and Tris. The reaction was started by the addition of enzyme and incubated at 30°C for 10 min. The reaction was stopped by addition of 0.1 ml of 1 M $HClO_4$. After neutralization with 5 M KOH, the reaction mixture was diluted with 1 ml of 0.1 M glycylglycine, pH 8.5, and centrifuged to remove particulate matter.

The reaction product, 5′-AMP, was separated by a modification of the method of Davis and Daly (6). Samples were poured onto small columns (0.7 by 2 cm) of Affi-Gel 601 (phenylboronate) positioned directly over scintillation vials. The columns were eluted with 7 ml of 0.1 M glycylglycine to remove unreacted cAMP. The 5′-AMP, which binds to the columns, was then eluted into separate vials with 8 ml of 0.1 M sodium acetate, pH 4.8. Radioactivity in both sets of vials was measured in a scintillation counter after addition of 10 ml of Scinti-Verse E. The ratio of product counts to total counts recovered gives the fractional hydrolysis of cAMP and was used to calculate the amount of product produced.

TABLE 1. Subcellular distribution of enzyme activity in *B. japonicum* bacteroids[a]

Bacteroid fraction	Adenylate cyclase (pmol)		Phosphodiesterase (pmol)	
	−SDS	+SDS	−SDS	+SDS
Homogenate	152 ± 2	650 ± 7	12.9 ± 0.6	6.1 ± 0.4
Soluble	18 ± 1	2 ± 1	6.7 ± 0.3	1.9 ± 0.2
Particulate	93 ± 5	470 ± 9	5.8 ± 0.4	5.5 ± 0.6

[a] Soluble and particulate fractions were prepared from a French pressure cell-ruptured bacteroid preparation (homogenate) by centrifugation at 144,000 × *g* for 2 h in a Beckman Ti 50 rotor. The pellet was suspended in the original volume of buffer. Enzyme assays were performed with the same volume of each sample (25 μl for adenylate cyclase and 10 μl for phosphodiesterase). Rates are reported as picomoles of product formed in 10 min and are the average of triplicate determinations ± the standard deviation. SDS was present at a concentration of 0.01%.

Acetylene reduction assays. Acetylene reduction assays, as a measure of nitrogen fixation activity, were performed by the method of Schwinghamer et al. (22).

Protein determination. The protein content of samples was measured by the method of Lowry et al. (15), with crystalline bovine serum albumin as a standard.

Electron microscopy. Samples of disrupted bacteroids were fixed in suspensions with 2% (vol/vol) gluteraldehyde. After incubation for 1 h on ice, the samples were sedimented by centrifugation at 220,000 × *g* for 1 h in a Beckman 50 Ti rotor. The pellets were postfixed with 1% OsO_4 and dehydrated in a graded series of ethanol prior to embedding. Ultrathin sections were stained with uranyl acetate and lead acetate.

Chemicals. Tris, sucrose, phosphocreatine, creatine phosphokinase, Triton X-100, cetyltrimethylammonium bromide (CTAB), CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonic acid), cholic acid, Gpp(NH)p (5′-guanylylimidodiphosphate), and other common reagents and salts were obtained from Sigma Chemical Co. [α-^{32}P]ATP and [2,8-^{3}H]cAMP were purchased from ICN Pharmaceuticals Inc.; Forskolin, MES, and HEPES were from Calbiochem-Behring; Affi-Gel 601 and sodium dodecyl sulfate (SDS) were from Bio-Rad Laboratories; and Scinti-Verse E scintillation fluid was from Fisher Scientific Co. Polyclar AT (PVP) was purchased from GAF Corp. Before use, the PVP was washed with 10% HCl, neutralized, washed with distilled water and dried.

RESULTS

The presence of adenylate cyclase and phosphodiesterase in soybean root nodules was determined by assaying the bacteroids and the plant cytosol at the various stages of the bacteroid isolation procedure. The only detectable adenylate cyclase activity was found in the bacteroid fraction; no significant activity was measured in the plant cytosol, plant mitochondria or plant membranes. The activity increased at least 10-fold after the bacteroids were ruptured in the French press, demonstrating that adenylate cyclase was located

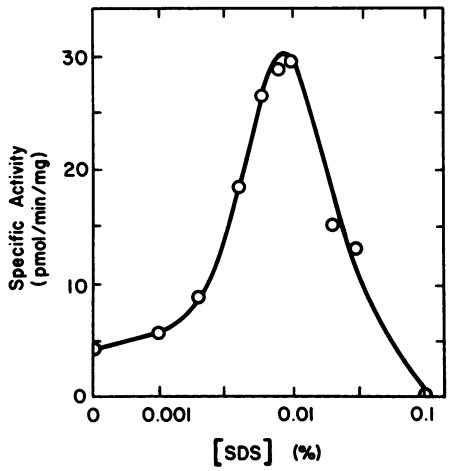

FIG. 1. Effect of SDS on adenylate cyclase activity in *B. japonicum* bacteroids. Bacteroid membrane preparations and adenylate cyclase assays are described in Materials and Methods.

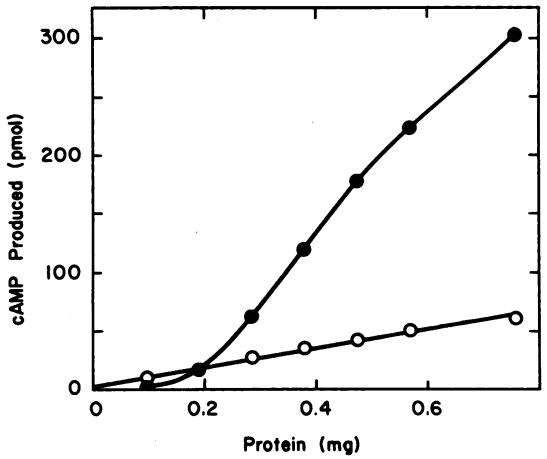

FIG. 2. Effect of protein content on adenylate cyclase activity of *B. japonicum* bacteroids in the presence and absence of SDS. Adenylate cyclase activity was measured as described in Materials and Methods in the absence (○) and presence (●) of 0.01% SDS. Activity is reported as picomoles of cAMP formed in 10 min.

within the bacteroid, primarily associated with the membrane (Table 1).

Bacteroid adenylate cyclase activity was found to be greatly increased when assayed in the presence SDS (Table 1). A typical activation curve for SDS is given in Fig. 1. The activity increased with increasing detergent concentration up to 0.01% SDS, followed by a rapid loss of activity at higher concentrations, presumably due to denaturation. Activation was not the result of solubilization, as the enzyme remained in the particulate fraction. Several other detergents were tested over a concentration range of 0.1 to 0.001%. Triton X-100 increased activity 2.4-fold, while cholate and CHAPS gave lesser activation (Table 2). Cetyltrimethylammonium bromide, a strong cationic detergent, showed no activation. Activation did not require preincubation of the enzyme sample with SDS, nor did preincubation alter the degree of activation.

The degree of activation by SDS varied with enzyme concentration in the assay. With a constant SDS concentration, the increase in activity with increasing protein concentration was proportional only at higher amounts of enzyme (Fig. 2). At lower amounts of enzyme, less activation was observed than expected, probably due to denaturation of the enzyme because of the greater relative amount of SDS, i.e., higher SDS/protein ratio (Fig. 1).

TABLE 2. Detergent activation of *B. japonicum* bacteroid adenylate cyclase[a]

Addition	cAMP formed (pmol)	Relative activity vs control
None (control)	47 ± 1	1.0
SDS (0.01%)	380 ± 17	8.1
Triton X-100 (0.1%)	112 ± 5	2.4
Cholate (0.1%)	80 ± 5	1.7
CHAPS (0.1%)	65 ± 2	1.4
None (control)	70 ± 5	1.0
CTAB[b]		
0.0001%	75 ± 5	1.1
0.001%	65 ± 8	0.9
0.01%	8 ± 1	0.1

[a] French pressure cell-ruptured bacteroid membrane preparations were assayed for adenylate cyclase as described in Materials and Methods with the indicated concentration of detergent. Product formation is given as picomoles of cAMP produced during a 10-min incubation at 30°C (average of triplicate determinations ± the standard deviation).

[b] CTAB, Cetyltrimethylammonium bromide.

Electron micrographs of bacteroid membrane preparations were taken to determine the effect of SDS on the physical integrity of the membranes. After French press treatment, the preparation contained many large, membrane-bound structures along with smaller membrane fragments and polyhydroxybutyrate granules (Fig. 3A). SDS treatment caused more complete disruption of the membranous structures, resulting in a uniform appearance of the membrane preparation (Fig. 3B).

The presence of SDS affected the kinetic constants of the enzyme with regard to MgATP. Double-reciprocal plots of velocity versus MgATP concentration, with 15 mM Mg^{2+}, gave K_m values of 1.6 and 0.47 mM and V_{max} values of 94.3 and 14.7 pmol/min per mg of protein in the presence and absence of 0.01% SDS, respectively.

Other properties of bacteroid adenylate cyclase appear not to be greatly affected by detergent activation. The enzyme had a pH optimum between 7.5 and 8.0 (Fig. 4A) and required a divalent metal ion for activity. Either Mg^{2+} or Mn^{2+}, but not Ca^{2+}, supported activity; no activity was found in the absence of Mg^{2+} or Mn^{2+}. At concentrations below 15 mM, Mn^{2+} supported greater adenylate cyclase activity than Mg^{2+}. Higher concentrations of Mn^{2+} were inhibitory. The K_{act} values for Mn^{2+} and Mg^{2+} were 1.0 and 1.4 mM, respectively.

To explore possible regulation of bacteroid adenylate cyclase, a wide variety of compounds were tested for their ability to alter enzyme activity. Compounds tested included Gpp(NH)p, forskolin, fluoride, glutamate, glutamine, hydroxybutyrate, pyruvate, pyrophosphate, phosphoenolpyruvate, Ca^{2+}, NH_4^+, NAD^+, and NADH. None of these compounds significantly affected enzyme activity.

The localization of cAMP phosphodiesterase activity within soybean root nodules was similar to that of adenylate cyclase, with most of the activity found within the bacteroids. However, phosphodiesterase activity was present in both the soluble and particulate fractions of French press-ruptured bacteroids (Table 1). The soluble enzyme was

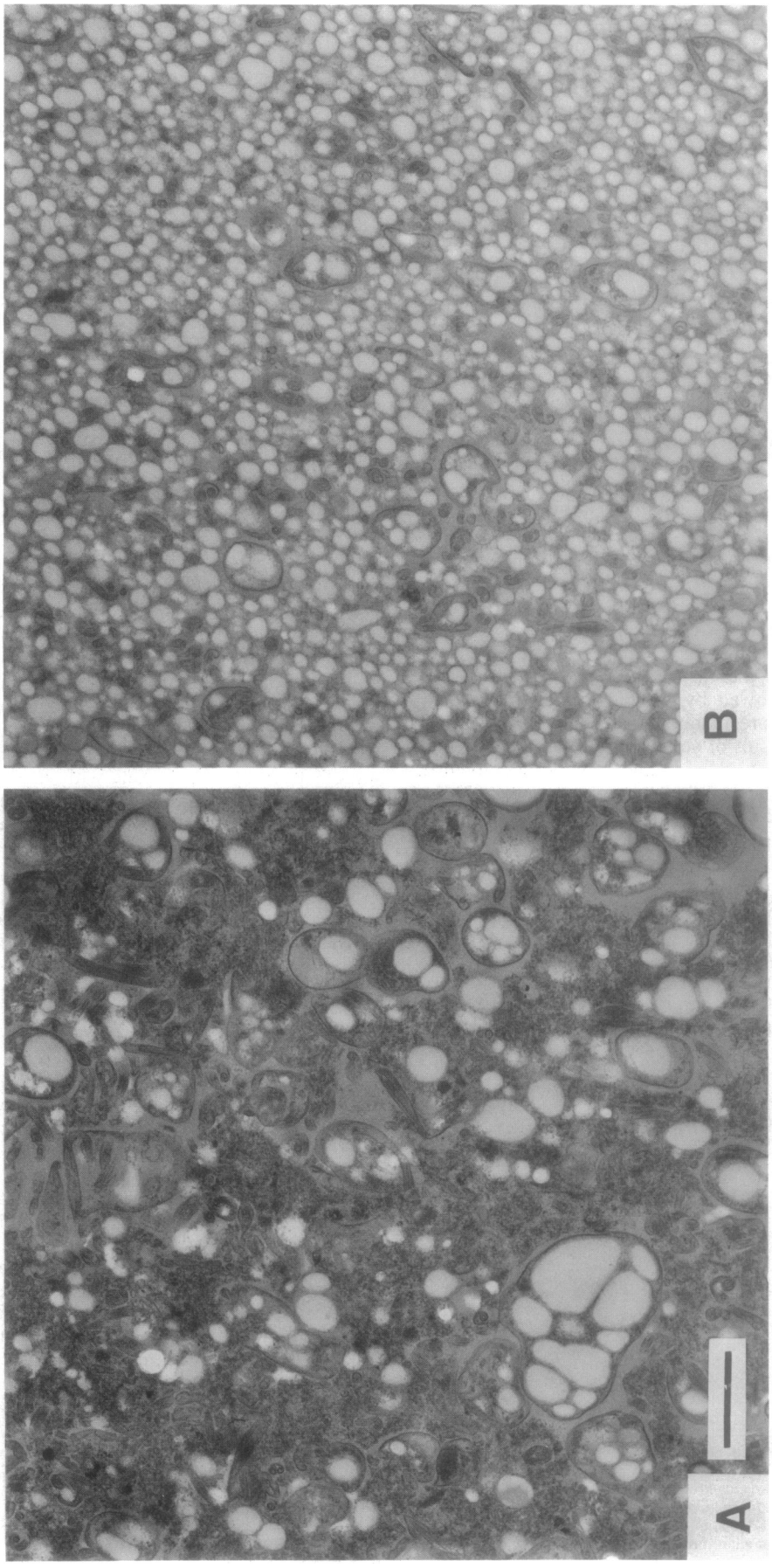

FIG. 3. Electron micrographs of SDS-treated and untreated, ruptured *B. japonicum* bacteroids. (A) Untreated, ruptured bacteroids. (B) Ruptured bacteroids treated with 0.01% SDS. Bar, 1 μm. Samples were processed as described in Materials and Methods.

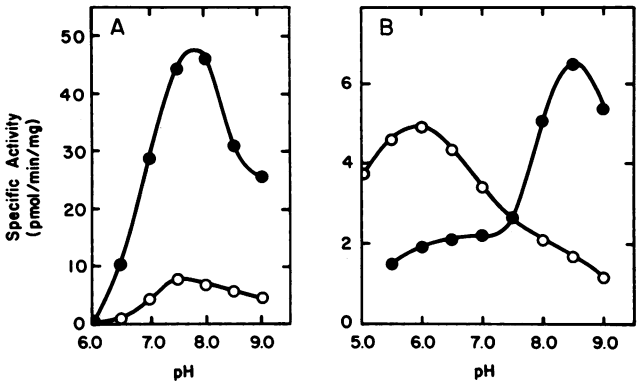

FIG. 4. pH dependence of adenylate cyclase and phosphodiesterase of *B. japonicum* bacteroids. (A) Adenylate cyclase activity in the absence (○) and presence (●) of 0.01% SDS. (B) Soluble (●) and particulate (○) activities of phosphodiesterase. Enzyme assays were performed as described in Materials and Methods.

inhibited more than 70% by 0.01% SDS, whereas the particulate fraction was inhibited less than 5% under the same conditions. Enzyme activity in the soluble fraction had a pH optimum near 8.5, but the particulate enzyme was most active at pH 6.0, suggesting that two distinct forms of this enzyme are present in bacteroids (Fig. 4B).

The two forms of phosphodiesterase also showed different responses to divalent metal ions. The soluble enzyme had an absolute requirement for a metal ion, with either Mg^{2+} or Mn^{2+} supporting activity. No activity was detectable in the presence of excess EDTA. The particulate enzyme, however, was active in the presence of EDTA, and the addition of Mg^{2+} only doubled the activity. The K_{act} for Mg^{2+} was 2.2 and 10.5 mM for the soluble and particulate phosphodiesterase, respectively. Both obeyed Michaelis-Menten kinetics when the cAMP concentration was varied, giving K_m values of 7.3 μM for the soluble enzyme and 3.4 μM for the particulate enzyme.

To gain further insight into the possible function of cAMP, bacteroids were isolated from nodules at several different ages and the specific activities of adenylate cyclase and phosphodiesterase were measured (Table 3). The specific activity of adenylate cyclase was low at day 17, increased until day 29, and appeared to remain nearly constant thereafter. Activation of the enzyme by SDS occurred at all time points; however, greater activation was seen on days 29 and 37. In contrast, the specific activity of phosphodiesterase, whether assayed at pH 8.5 or 6.0, was initially high and decreased with nodule age. This decrease was most pronounced at pH 6.0, at which the activity of the particulate enzyme predominates, suggesting that this form of the enzyme was changing with nodule age.

TABLE 3. Enzyme activity of *B. japonicum* bacteroids during nodule development[a]

Time post-inoculation (days)	Activity (pmol/min per mg of protein)				
	Adenylate cyclase		Phosphodiesterase		Acetylene reduction (μmol/h per g)
	−SDS	+SDS	pH 6.0	pH 8.5	
17	9 ± 0.2	44 ± 3	21 ± 5	9.5 ± 2.5	8.5 ± 1.2
21	24 ± 1.9	95 ± 13	18 ± 3	9.3 ± 1.9	25.2 ± 3.4
29	32 ± 1.8	209 ± 11	19 ± 1	9.8 ± 0.6	18.2 ± 7.6
37	22 ± 1.8	215 ± 11	13 ± 1	6.9 ± 0.9	5.8 ± 4.3

[a] Soybean nodules were obtained at the times indicated after inoculation with *B. japonicum*. Bacteroids were isolated from the nodule tissue as described in Materials and Methods and ruptured by passage through a French pressure cell. When present, SDS was used at a concentration of 0.01%. Units of acetylene reduction activity are micromoles of ethylene formed per hour per gram (fresh weight) of nodules. All values are averages of triplicate determinations ± the standard deviation.

DISCUSSION

The presence of enzymes for the synthesis and hydrolysis of cAMP was demonstrated in bacteroids of *B. japonicum*. Within the bacteroid, adenylate cyclase activity was firmly bound to the particulate portion of the cell and was not released after treatment with detergent. This contrasts with the properties of adenylate cyclase reported for other microorganisms, in which the enzyme was either soluble or loosely associated with particulate material (1, 4, 10, 18, 24, 29). During attempts to solubilize the enzyme, it was observed that SDS, at low concentrations, increased enzyme activity four- to fivefold. This activation was highly concentration dependent, did not require preincubation, and was not accompanied by release of the enzyme into solution. Of the detergents tested, only SDS gave substantial activation. The reason for increased adenylate cyclase activity is not clear. However, SDS treatment resulted in more complete disruption of the membranes, which perhaps allowed a better enzyme-substrate interaction, caused a structural change in which latent enzyme was exposed, or possibly removed a strongly held regulatory molecule. The lack of a similar increase in activity with other detergents, particularly cetyltrimethylammonium bromide, indicates involvement of the negative charge. On the other hand, lack of activation by other polyanions demonstrates the importance of the detergent properties of SDS. Apparently, a strong, negatively charged detergent is needed. However, other bacteroid enzymes did not show large increases in activity in the presence of SDS, suggesting relatively efficient exposure of "soluble" enzymes by French press treatment. These include soluble enzymes such as malate dehydrogenase and both forms of phosphodiesterase.

The cAMP phosphodiesterase activity of bacteroids was found in both the particulate and the soluble fractions. The two enzyme forms had distinct pH optima and differed in their response to divalent metal ions and SDS treatment. The soluble enzyme has a high pH optimum, which is typical of other enzymes found inside the bacteroid (11), and is markedly inhibited by SDS. The particulate enzyme, in contrast, has an acidic pH optimum, shows substantial activity in the absence of divalent metal ion, and is unaffected by the presence of SDS. The bacteroid therefore appears to contain two different activities which catalyze hydrolysis of cAMP.

Adenylate cyclase activity increased and phosphodiesterase activity declined during the increase in acetylene reduction activity, indicating that the level of cAMP increases during the development of symbiotic nitrogen fixation. There are several observations concerning cAMP in procaryotes relevant to symbiotic nitrogen fixation. First, cAMP levels correlate with morphological changes in dimorphic organisms (2). During symbiotic development, the endophyte undergoes morphological and biochemical changes to the bacteroid form (23). Second, intracellular cAMP levels increase at the onset of general carbon starvation, specifically with glucose depletion (2). The bacteroid is unable to transport and metabolize glucose and other carbohydrates (23). Third, there appears to be an inverse correlation between cAMP levels and the energy status of the organism (2). The

high demand for energy by the nitrogen fixation process lowers the energy charge of the cell (27). Fourth, cAMP additions repress the ammonia-assimilatory enzymes of free-living cultures of *B. japonicum* (25). The activities of ammonia-assimilatory enzymes of the bacteroid are too low to assimilate a significant amount of ammonia produced by nitrogenase (23). Although these examples are only correlations, they provide possibilities as to the role(s) of cAMP in symbiotic nitrogen fixation. Recently, the presence of two different genes for adenylate cyclase in *B. japonicum* has been reported (3), raising the possibility of multiple functions for this enzyme, perhaps related to the symbiotic and free-living forms. Additional research is needed to identify these functions and their relationship to the different forms of the organism.

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